Remarks

Claims 16-20 are canceled without prejudice as being non-elected. Claims 21-31 are added. Thus, claims 1-15 and 21-31 remain pending. Claims 3, 9, 11-13, and 15-20 were withdrawn from consideration, but the inclusion of claim 12 in prosecution is respectfully requested. Claim 12 is amended to clarify that elected SEQ ID NO:34 provides the sequence for the XptA2 x_{wi} protein.

Claims 1, 2, 4-8, 10, and 14 stand rejected for lack of an adequate written description. The office action states that no variants or truncation fragments are disclosed in the specification and that one skilled in the art would have no idea which variants or fragments are included within the scope of the claims. The applicants respectfully traverse this rejection.

It should be noted that a wide range of potentiators (B and C proteins) were successfully used according to the subject invention with both a *Photorhabdus* Protein A toxin and with a *Xenorhabdus* Protein A toxin. Thus, many representative species within the genus were disclosed.

It should be clear that the claims do not refer to any fragment or variant of the specified sequences. Rather, a range of variants, with certain activities, is specified. To provide further clarification in these regards, claim 1 specifies that the Proteins retain functional activity. (*See* paragraph 158, for example, of the specification.) It should be clear that inactive fragments and the like are not included.

One skilled in the art does know which variants and fragments are within the scope of the claimed invention. Regarding fragments of toxin complex (TC) proteins, the Background section (paragraph 21) of the specification states, "It was determined that many of these gene products were cleaved by proteases. For example, both TcbA and TcdA are cleaved into three fragments termed i, ii and iii (*e.g.* TcbAi, TcbAii and TcbAiii). Products of the *tca* and *tcc* ORFs are also cleaved. *See* **Figure 7.** *See also* R.H. ffrench-Constant and D.J. Bowen, *Current Opinions in Microbiology,* 1999, 12:284-288. Attached is a copy of this reference.

Figure 1 of this reference clearly shows, and the caption states that vertical arrows indicate, "...the predicted proteolytic cleavage sites present in both *Photorhabdus* and *Xenorhabdus* toxins..." Note that orf2 and orf5 of *Xenorhabdus* (for proteins XptA1 and

XptA2) are very similar in this regard to tcbA and tcdA of *Photorhabdus*. Regarding this reference, paragraph 36 of the specification further states,

> Analysis of the sequence in that cosmid suggested that there are five different ORF's with similarity to *Photorhabdus tc* genes; *orf2* and *orf5* both have some level of sequence relatedness to both *tcbA* and *tcdA,* whereas *orf1* is similar to *tccB, orf3* is similar to *tccC* and *orf4* is similar to *tcaC*. A number of these predicted ORFs also share the putative cleavage site documented in *P. luminescens*, suggesting that active toxins might also be proteolytically processed.

Paragraph 30 of the specification states

> Toxin A is comprised of two different subunits. The native gene tcdA encodes protoxin TcdA. As determined by mass spectrometry, TcdA is processed by one or more proteases to provide Toxin A. More specifically, TcdA is an approximately 282.9 kDa protein (2516 aa) that is processed to provide TcdAi (the first 88 amino acids), TcdAii (the next 1849 aa; an approximately 208.2 kDa protein encoded by nucleotides 265-5811 of *tcdA*), and TcdAiii, an approximately 63.5 kDa (579 aa) protein (encoded by nucleotides 5812-7551 of *tcdA*). TcdAii and TcdAiii appear to assemble into a dimer (perhaps aided by TcdAi), and the dimers assemble into a tetramer of four dimers.

In addition, U.S. Patent No. 6,528,484 states [underlining added]:

> As is further illustrated in the Examples, the tcbA gene was expressed in E. coli as two possible biological active protein fragments (TcbA and TcbAii/iii). The tcda gene was also expressed in E. coli. <u>As illustrated in Example 16, when the native unprocessed TcbA toxin was treated with the endogeneous metalloproteases or insect gut contents containing proteases, the TcbA protein toxin was processed into smaller subunits that were less than the size of the native peptides and Southern Corn Rootworm activity increased</u>. The smaller toxin peptides remained associated as part of a toxin complex. It may be desirable in some situations to increase activation of the toxin(s) by proteolytic processing or using truncated peptides. Thus, it may be more desirable to use truncated peptide(s) in some applications, i.e., commercial transgenic plant applications.

Paragraph 28 of the subject application cites WO 00/30453 and WO 00/42855 as disclosing TC-like proteins from *Xenorhabdus*. Example 7 of WO 00/42855 is entitled "Fragments that encode nematocidal activity" and discusses active fragments of *Xenorhabdus* toxins.

Still further, pages 14-17 of WO 00/42855 and pages 7-14 of WO 00/30453 discuss the use of variants, including those with conservative amino acid substitutions, of *Xenorhabdus* proteins.

In addition, the naturally occurring members of each class of proteins specified in the subject claims span a range of sizes. Thus a longer version could be truncated to mimic a shorter version, and then tested for functional activity. This would provide direction to one skilled in the art as to what types of truncations and substitutions to make.

It is common knowledge, and common practice, in the *Bacillus thuringiensis (B.t.)* art to use, and to claim, an insecticidal *Cry* protein and insecticidal fragments thereof. A wide variety of fragments of a typical 130 kDa *B.t.* insecticidal protein can be constructed. *See e.g.* U.S. Patent No. 5,710,020; 6,114,138; 6,229,004; and 6,251,656. Paragraph 186 of the specification further states,

> It is well-known in the art that truncated toxins can be successfully produced so that they retain functional activity while having less than the entire, full-length sequence. It is well known in the art that *B.t.* toxins can be used in a truncated (core toxin) form. *See, e.g.*, Adang *et al.*, *Gene* 36:289-300 (1985), "Characterized full-length and truncated plasmid clones of the crystal protein of *Bacillus thuringiensis* subsp *kurstaki* HD-73 and their toxicity to *Manduca sexta.*" There are other examples of truncated proteins that retain insecticidal activity, including the insect juvenile hormone esterase (U.S. Pat. No. 5,674,485 to the Regents of the University of California). As used herein, the term "toxin" is also meant to include functionally active truncations.

Thus, once a typical full-length toxin is disclosed, one skilled in the art could make a wide range of fragments of the toxin and test such fragments to confirm that they retain insecticidal activity.

It is also noted that many of the amino acids listed Table 1, for example, following paragraph 191 of the specification, are very similar in structure to each other, with many differing only by a single carbon group. This is the case for alanine compared to glycine, and serine and threonine, for example. Isoleucine and leucine, and valine and proline, have the same number of carbon atoms but differ only in the arrangement of the carbon atoms in the sidechain. Serine and threonine, asparagine and glutamine, and aspartic acid and glutamic acid likewise differ by only a single carbon group. One having even *some* skill in the art could simply go through SEQ ID NO:34, for example, and make such substitutions at 10% (for example) of the

residues, and then test these products for insecticidal activity (using protocols and assays that are well-known in the art). Of the first 30 amino acid residues of SEQ ID NO:34, for example, only eight of them are not residues discussed in the preceding paragraph.

The applicants also wish to note that the hybridization language used in claims 21-31 is generally accepted as satisfying the written description requirements, and the specification as filed clearly provided support for these claims. *See* Examples 9-10, for example, of the Written Description Guidelines.

In light of all the foregoing, it should be clear that the applicants had possession of the invention now claimed. Thus, the withdrawal of the rejection for lack of adequate written description is respectfully requested.

Claims 1, 2, 4-8, 10, and 14 stand rejected for lack of enablement. The applicants respectfully traverse this rejection.

The comments above regarding the written description are fully applicable to this issue, as well. As discussed above, active fragments of *Xenorhabdus* and *Photorhabdus* toxins were known in the art and are discussed in the specification. A wide range of potentiators (B and C proteins) were successfully used according to the subject invention with both a *Photorhabdus* Protein A toxin and with a *Xenorhabdus* Protein A toxin. The subject application contains many, many Examples, and many, many data Tables reporting a very large number of various combinations of proteins that were used according to the subject invention. Furthermore, the use of a range of variants, once proteins are exemplified, is reasonable and is known in the art, as evidenced by the Horticulture Research International patent applications and other art discussed above.

In addition, the naturally occurring members of each class of proteins specified in the subject claims span a range of sizes. Thus a longer version could be truncated to mimic a shorter version, and then tested for functional activity. This would provide direction to one skilled in the art as to what types of truncations and substitutions to make.

It is again noted that one skilled in the art can make conservative amino acid substitutions, and that many amino acids are very similar in structure. This is discussed in more detail above. Again, only 8 of the first 30 residues of SEQ ID NO:34 do <u>not</u> fall into one of

those categories. Thus, one having even *some* skill in the art could produce and test a wide range of variants based on SEQ ID NO:34. While many variants within the scope of the claims could be designed, produced, and tested, such experimentation in this context (as opposed to an emerging field, such as anti-sense technology for example) should not be considered "undue." A considerable amount of experimentation is permissible if it is merely routine. In re Wands 858 F2d 731, 737, 8 USPQ2d 1400, 1404. A considerable amount of time and expense are also permissible. United States v. Telectronics Inc. 857 F2d 778, 8 USPQ2d 1217 (Fed. Cir. 1988).

In light of all the foregoing, the withdrawal of the rejection for lack of enablement is respectfully requested.

Claims 1, 2, 4-8, 10, and 14 stand rejected for indefiniteness. The applicants wish to thank the Examiner for suggesting that conservative amino acid substitutions be specified in the claims, in accordance with paragraph 190 of the specification. In addition, hybridization conditions are added to the relevant claims, in accordance with the Examiner's suggestion. In light of all the foregoing, the withdrawal of the indefiniteness rejections is respectfully requested.

Claims 1, 2, 4-8, 10, and 14 stand rejected for obviousness. The applicants respectfully traverse this rejection.

[0001] Paragraph 43 of the subject specification states, "While *Photorhabdus* toxins have been used successfully, and *Xenorhabdus* toxins have been used successfully (apart from *Photorhabdus* toxins), enhancing the activity of a TC protein toxin from one of these source organisms (such as a *Photorhabdus*) with one or more TC protein potentiators from the other (a *Xenorhabdus,* for example) has not heretofore been proposed or demonstrated." The statements made in the specification have the effect of an expert declaration by each of the skilled inventors, who executed the oath/declaration.

Paragraph 44 of the specification states,

> The subject invention relates to the surprising discovery that toxin complex (TC) proteins, obtainable from organisms such as *Xenorhabdus*, *Photorhabdus*, and *Paenibacillus*, can be used interchangeably with each other. As one skilled in the art will recognize with the benefit of this disclosure, this has broad implications and expands the range of utility that individual types of TC proteins will now be recognized to have. This was not previously contemplated, and it would not have been thought possible, especially given the high level of divergence at the

> sequence level of the TC proteins from *Photorhabdus* compared to 'corresponding' TC proteins of *Xenorhabdus* and *Paenibacillus,* for example.

Although *Photorhabdus* and *Xenorhabdus* might share some common ancestry, given the level of sequence divergence, for example, among the various proteins that each of these strains produce, one simply could not have predicted that mixing these proteins would allow for, for example, proper interaction and binding of the different components to form the toxin complex. As stated in paragraph 128 of the specification, "It is in this context that it was discovered, as described herein, that *Xenorhabdus* TC proteins could be used to enhance the activity of *Photorhabdus* TC proteins and *vice versa. Paenibacillus* TC proteins are also surprisingly demonstrated herein to potentiate the activity of *Xenorhabdus* (and *Photorhabdus*) TC toxins. This was not previously proposed or demonstrated, and was very surprising especially in light of the notable differences between *Xenorhabdus*, *Photorhabdus*, and *Paenibacillus* TC proteins. There was certainly no expectation that divergent proteins from these divergent organisms would be compatible with each other."

Paragraph 155 further elaborates [underlining added]:

> The subject invention also provides 'mixed pairs' of potentiators such as Potentiator Pairs 3, 4, and 5 as illustrated above. Such combinations were not heretofore expected (or suggested) to be active as TC protein toxin enhancers. Thus, such 'heterologous' combinations of potentiators can now be selected to maximize their ability to enhance two (for example) insecticidal toxins. That is, one might now find that, for a given use, TcdB1 and XptB1 is a more desirable pair of potentiators than is XptC1 and XptB1, for example. Again, this is surprising given the relative degree of sequence divergence between a given *Photorhabdus* potentiator and a *Xenorhabdus* potentiator for which it is substituted, as well as the degree of difference between the natural 'target' toxins which the potentiators would naturally enhance. Therefore, it should be clear that the subject invention also provides heterologous potentiator pairs (*i.e.,* where the Class B (~170 kDa) potentiator is derived from a bacterial genus that is different from the bacterial genus from which the Class C (~112 kDa) potentiator is derived).

In light of all the foregoing, it should be clear that the obviousness rejection involves hindsight reconstruction, which is improper. It cannot possibly be maintained that the art provided an expectation of success that the surprising new combinations of proteins would

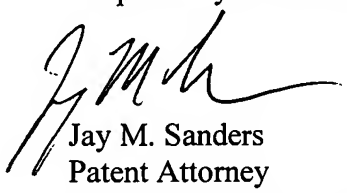
function together in advantageous new ways. Thus, the withdrawal of the rejection for obviousness is respectfully requested.

The applicants believe that this application is in condition for allowance, and such action is earnestly solicited.

The Assistant Commissioner is hereby authorized to charge any fees under 37 CFR §§1.16 and 1.17 as required by this paper to Deposit Account 19-0065.

The applicants invite the Examiner to call the undersigned if clarification is needed on any of this response, or if the Examiner believes a telephonic interview would expedite the prosecution of the subject application to completion.

Respectfully submitted,



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Attachment: R.H. ffrench-Constant and D.J. Bowen, *Current Opinions in Microbiology,* 1999, 12:284-288.

Photorhabdus toxins: novel biological insecticides

Richard ffrench-Constant* and David Bowen†

Following concerns over the potential for insect resistance to insecticidal *Bacillus thuringiensis* toxins expressed in transgenic plants, there has been recent interest in novel biological insecticides. Over the past year there has been considerable progress in the cloning of several alternative toxin genes from the bacteria *Photorhabdus luminescens* and *Xenorhabdus nematophilus*. These genes encode large insecticidal toxin complexes with little homology to other known toxins.

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Abbreviations

ORF	open reading frame
Tc	toxin complex

Introduction

With the widespread deployment of transgenic plants expressing insecticidal toxin genes from *Bacillus thuringiensis*, there has been considerable concern over the evolution of insect resistance to *B. thuringiensis* toxins [1,2]. One recent study has shown that resistant diamond back moths can completely overcome high levels of *B. thuringiensis* toxin expressed in transgenic canola [3•]. Several strategies have been suggested for prolonging the effective life of *B. thuringiensis* toxins including alternation with, or co-deployment off, different toxins [4]. The recent efforts to clone insecticidal toxin genes from the *Photorhabdus/Xenorhabdus* group of bacteria present potential alternatives to toxins derived from *B. thuringiensis*.

Photorhabdus and *Xenorhabdus* spp. are Gram-negative bacteria that form entomopathogenic symbioses with soil nematodes. These bacteria are found in the gut of entomopathogenic nematodes that invade and kill insects. When the nematode invades an insect host the bacteria are released into the insect haemocoel (the open circulatory system), and both the bacteria and the nematode undergo multiple rounds of replication whilst the insect host dies [5••]. These bacteria can be cultured away from their nematode hosts, and 50% insect mortality has been reported with direct infection of fewer than five bacteria per larva [6]. The bacteria secrete a wide variety of substances into the culture medium including lipases, proteases, antibiotics and lipopolysaccharides [7–13]. Several of these substances have previously been implicated in insect toxicity but until recently no insecticidal genes had been cloned.

In the past year there has been substantial progress in the cloning of genes encoding insecticidal toxins both from *Photorhabdus luminescens* and *Xenorhabdus nematophilus*. This review focuses on the cloning of the toxin complex encoding genes from *P. luminescens* and then draws parallels with the genes more recently cloned from *X. nematophilus*.

Photorhabdus toxin purification

Previous studies had suggested that the insecticidal effects of *P. luminescens* may involve secreted proteases, lipases or lipopolysaccharides. Protein purification work by Bowen and Ensign [14••], however, clearly separated a high molecular weight fraction of the bacterial culture medium showing oral and injectable toxic activity against a range of insects. This fraction had an estimated molecular weight of 1,000,000 and contained no protease, phospholipase or hemolytic activity, suggesting a novel activity. Insecticidal activity could be eliminated by heat treatment and was also neutralized by a polyclonal antibody raised against the mixture of complexes from the high molecular weight fraction. Subsequently this fraction was separated by high performance liquid chromatography (HPLC) into four different toxin complexes (Tcs) Tca, Tcb, Tcc and Tcd [15••]. Bioassay of one of these toxin complexes, Tca, revealed it to be highly toxic to first instar tomato hornworms (*Manduca sexta*) when given orally and to have a median lethal dose (LD^{50}) of 875 ng per square centimeter of artificial diet [15••]. This is equivalent to some of the less active *B. thuringiensis* toxins [16]. Feeding, however, was inhibited at Tca doses as low as 40 ng/cm^2. Further, given the high predicted molecular weight of Tca, on a molar basis, *P. luminescens* toxins are more active and relatively few molecules are necessary to exert a toxic effect. Each of these toxin complexes resolves as either a single or dimeric species on a native agarose gel but resolution on a denaturing gel reveals that each complex consists of a range of species between 25–200 kDa for which amino-terminal amino acid sequence was determined by protein sequencing [15••].

Toxin complex gene cloning

In order to clone the toxin complex (*tc*) genes, genomic libraries of *P. luminescens* were screened with both monoclonal and polyclonal antibodies raised against the toxins. Four *tc* loci were cloned: *tca*, *tcb*, *tcc* and *tcd* (Figure 1) [15••]. *tca* and *tcc* are operons of three open reading frames (ORFs) transcribed in the same direction (*tcaA*, *tcaB* and *tcaC*) with a smaller terminal ORF transcribed in the opposite direction (*tcaZ*). The other two operons (*tcb* and *tcd*) are both single long ORFs. Comparison of the amino-terminal sequences derived from polypeptides found in the culture medium with sequences predicted from the *tc* ORFs shows that many of gene products are subsequently cleaved. For example, both Tcb and Tcd are cleaved into three fragments termed i,

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ii and iii (e.g. Tcbi, Tcbii and Tcbiii). Products of the *tca* and *tcc* ORFs also cleaved (Figure 1).

Interestingly none of the four loci show overall similarity to any sequences currently deposited in GenBank. Regions of TcaC and TccA, however, show similarity to the SpvA and SpvB proteins, respectively. *Salmonella* plasmid virulence (*spv*) genes are required for the growth of *Salmonella dublin* in bovine macrophages [17•]. *spvB* in particular is required for *S. typhimurium* to induce cytopathology in human monocyte-derived macrophages (D Guiney, personal communication). Similarity between virulence proteins from bacteria that invade insects and vertebrates is potentially highly significant and also raises the suggestion that in *P. luminescens* these proteins may overcome insect immunity by attacking insect hemocytes.

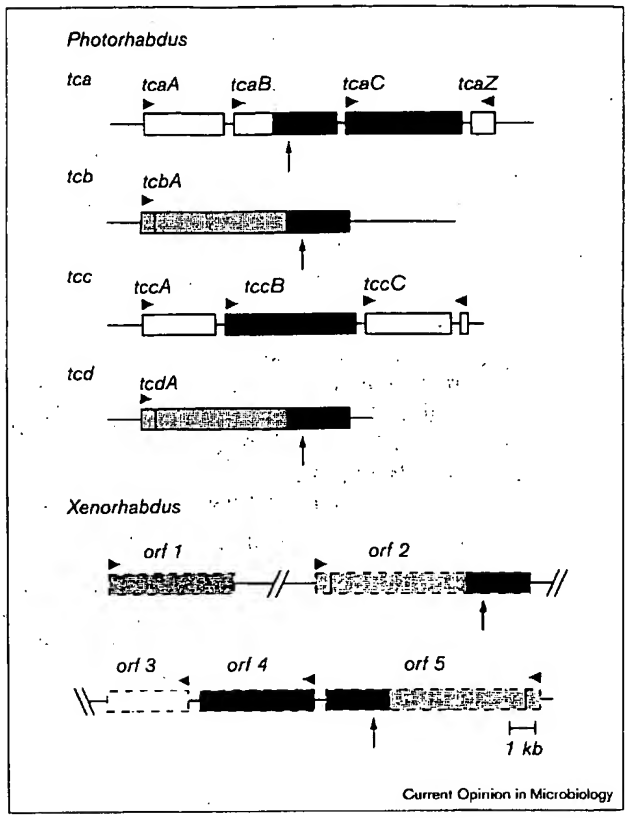
Despite the lack of similarity with other known sequences, there is considerable amino acid similarity between different predicted Tc proteins. Thus, TcaB, TcbA and TcdA all show amino acid conservation (53% identity) immediately around their predicted protease cleavage sites (Figure 2). This conservation between three different Tc proteins suggests that they may all be processed by the same or similar proteases. Tcb and Tcd also share 52% identity overall, as well as a similar predicted pattern of both carboxy- and amino-terminal cleavage, suggesting that they are homologs of one another. Further, *tcb* and *tcd* both encode very large proteins similar in size to the *Clostridium difficile* toxins A and B, although the overall percentage amino acid identity to either is only 17% [18]. The similarity of their large size and also the fact that both toxins appear to act on the gut of the organism [19] may suggest similar modes of action.

Toxin gene knockout mutants

To investigate the role of the *tc* genes in oral toxicity to insects both heterologous expression in *E. coli* and also gene knockout studies in *P. luminescens* itself were conducted [15••]. A wide range of *tc* constructs were expressed in *E. coli*, however, the corresponding polypeptides were neither secreted nor processed correctly and were not orally toxic to *M. sexta* [15••]. This is not unexpected given that active molecules appear cleaved in the culture medium and that this cleavage may be carried out by proteases also secreted by *P. luminescens* into the growth media (see below).

As a second approach, each of the *tc* loci in turn was either deleted or disrupted. This approach not only allows an assessment of the effects of deleting individual loci (i.e. *tca*⁻, *tcb*⁻, *tcc*⁻ and *tcd*⁻) but also the effects of deleting two loci together (e.g. *tca*⁻/*tcb*⁻). Deletion of either *tca* or *tcd* dramatically decreased the percentage mortality and correspondingly increased the relative weight gain of larvae placed on artificial diet treated with bacteria supernatant. Deletion, however, of both these loci, *tca*⁻/*tcd*⁻, completely abolished oral toxic activity

Figure 1



Map of the toxin complex (*tc*) loci in *Photorhabdus luminescens* and those predicted from the sequences of an orally toxic cosmid in *Xenorhabdus nematophilus*. In *P. luminescens* there are four loci termed *tca, tcb, tcc* and *tcd*. *tca* and *tcc* are operons with a similar genomic organization and *tcbA* and *tcdA* are single long ORFs with high identity to each other (see text for discussion). Following analysis of the published sequence for the *X. nematophilus* cosmid we predict that there are five ORFs with homology to *P. luminescens tc* genes. These ORFs, however, are outlined by dashed boxes to indicate that frame shifts between different predicted reading frames were observed in the sequence. The *Xenorhabdus* ORFs are shaded to indicate regions of homology with the *tc* genes: The vertical arrows indicate the predicted proteolytic cleavage sites present in both *Photorhabdus* and *Xenorhabdus* toxins, the sequence of which is shown in Figure 2. The horizontal arrowheads show the direction of transcription.

[15••]. This suggests that Tca and Tcd account for the majority of oral toxicity to lepidoptera. Interestingly, deletion of either *tcb* or *tcc* alone also reduces mortality, suggesting that there may be complex interactions among the different gene products. Thus, TcD may require TcA for enhancement of toxicity, in a fashion analogous to toxin B of *C. difficile*. Alternatively, TcD may modulate the toxicity of TcA and possibly other complexes. Finally it should be noted that all of the above discussions relate to oral activity against a single insect species. Therefore, *tcb* or *tcc* may produce toxins more active against other groups of insects or active via injection directly into the insect haemocoel (the normal route of delivery when secreted by the bacteria *in vivo*).

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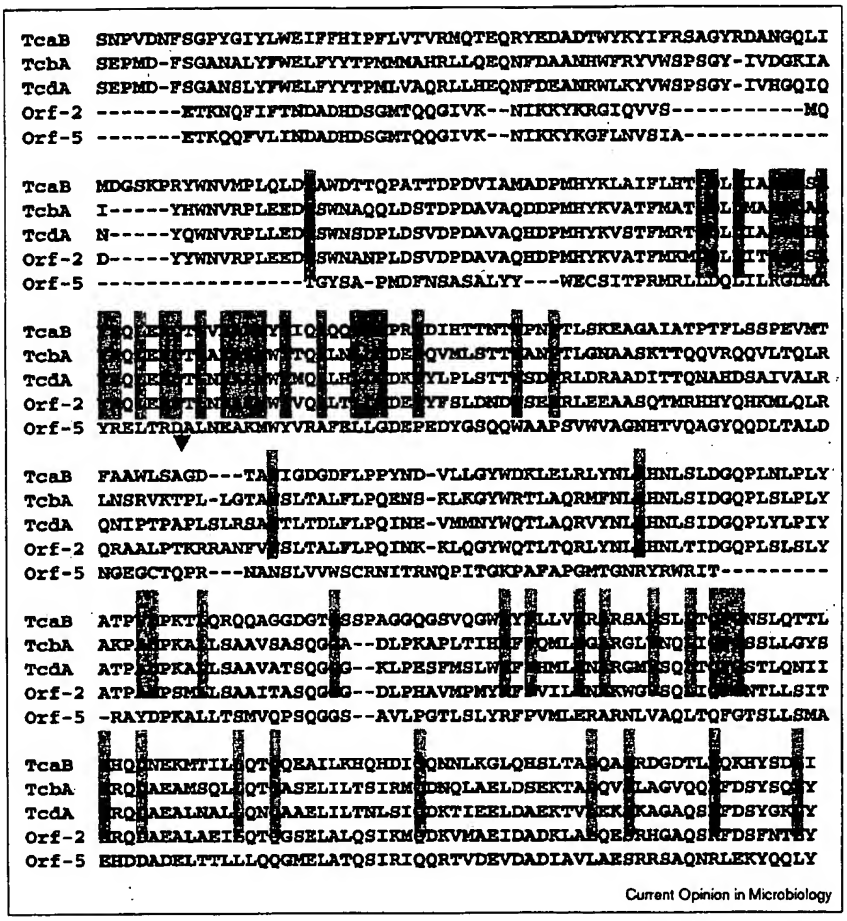
Figure 2



Current Opinion in Microbiology

Alignment of the predicted amino acid sequences of both *Photorhabdus* and *Xenorhabdus* tc-like toxins around the putative proteolytic cleavage site (arrow) in each polypeptide. Note how identity between the ORFs is high either side of, but reduced immediately around, the predicted cleavage site. The relative location of the different ORFs is shown in Figure 1.

Secreted proteases

In order to clarify the role of the secreted proteases either in direct insect toxicity and/or in toxin cleavage, a detailed purification of the different protease fractions present in the culture medium was carried out. Three fractions containing protease activity were resolved: one containing a single species of 56 kDa and two others (termed 40A and 40B) containing multiple, putatively related, species of approximately 40 kDa (Bowen D, Blackburn M, ffrench-Constant RH, unpublished data). These three fractions show different activities against different protease substrates and also different degrees of inhibition with a range of inhibitors supporting the assumption that they contain a number of different enzymes. These results are difficult to compare with those of other investigators due to differences in the strains used, the phase variants examined and their culture conditions [10,20].

Purification of the three different protease fractions, however, has clearly demonstrated that they are not involved in the oral toxic activity of *P. luminescens* culture medium, which clearly separates away with the Tc proteins only. Protease purification and separation will also facilitate an examination of their putative role in inhibiting antibacterial proteins such as cecropin [21•] and their potential in accounting for the patterns of cleavage observed in the purified high molecular weight Tc complexes [15••].

Histopathology of the insect midgut

Given that the toxin complexes must normally be secreted by the growing bacteria directly into the insect haemocoel, the oral activity of *P. luminescens* toxins is perhaps unexpected. In order to clarify the relationship between oral and injectable activity and to document the effects on the insect gut we looked at the histopathology of Tca on the *M. sexta* midgut [22••].

The insect midgut epithelium contains both columnar (structural) and goblet (secretory) cells. Ingestion of Tca leads to apical swelling and blebbing of large cytoplasmic vesicles by the columnar cells, leading to the eventual extrusion of cell nuclei in vesicles into the gut lumen. Goblet cells are also apparently affected in the same fashion, although we can not determine if the toxin is the proximal cause. Interestingly, Tca acts on the insect midgut following either oral delivery or injection [22••]. One important

difference in the histopathology of Tca injected larvae is the apparent absence of goblet cells in the anterior midgut. This may simply be due to their close proximity to the basal membrane in this region, whereas in the posterior midgut the goblet cells are completely enveloped by the columnar cells and, thus, may be shielded from direct toxin exposure from the haemocoel. This result was unexpected as is was not predicted that a toxin normally delivered into the insect haemocoel would show similar effects on the insect midgut following delivery to either side of the gut (via the gut lumen or the insect haemocoel).

This suggests two important working hypotheses for the mode of action of Tca. Firstly, that unlike some of the δ-endotoxins produced by *B. thuringiensis* [23], proteolytic processing of the toxin complex components by the insect midgut itself may not be necessary for normal toxin activity. Secondly, the ability of Tca to effect the midgut from either side suggests that the factors governing the interaction of toxin with insect cells either are relatively nonspecific or that the receptors for Tca are found on both the apical and basal surfaces of the midgut epithelium. The observation that other tissues exposed to the haemocoel are unaffected by toxin action would seem to preclude the possibility that the effects of Tca are nonspecific in relation to type of tissue attacked. Finally, although effects of Tca are seen on the midgut both via oral delivery and injection, we can not preclude the possibility that the primary site of action of this toxin *in vivo* is on another part of the insect. For example, Tca secreted into the haemocoel by the bacterium may also be designed to destroy insect hemocytes and thus overcome insect bacterial immunity.

In conclusion, broadly speaking, the histopathology of Tca action is similar to that seen for other novel gut active toxins such as the δ-endotoxins and Vip3A from *B. thuringiensis* [24–27] and also cholesterol oxidase [28]. This suggests that orally active toxins produce a similar range of histopathological effects on the insect midgut despite different presumptive modes of action, and, in the case of Tca, different modes of delivery.

Xenorhabdus homologs

Recent cloning efforts in *Xenorhabdus nematophilus* also appear to have identified novel insecticidal toxin genes with homology to the *P. luminescens tc* loci [P1••]. In this study cosmid clones were screened directly for oral toxicity to another lepidopteran *Pieris brassicae*. One orally toxic cosmid clone was sequenced [P1••]. Our analysis of the sequence in this cosmid suggests that there are five, different ORFs will similarity to *Photorhabdus tc* genes (Figure 1). Thus, *orf2* and *orf5* both have high identity to both *tcb* and *tcd*, whereas *orf1* is similar to *tccB*, *orf3* is similar to *tccC* and *orf4* is similar to *tccC*. Importantly, a number of these predicted ORFs also share the putative protease cleavage site documented in *P. luminescens* (Figure 2), suggesting that active toxins may also be proteolytically processed.

The finding of similar toxin encoding loci in these two different bacteria is interesting in terms of the possible origins of these virulence genes. The *X. nematophilus* cosmid also appears to contain transposase like sequences whose presence may suggest that these loci can be transferred horizontally between different strains or species of bacteria. A range of such transfer events may also explain the apparently different genomic organization of the *tc* operons in the two different bacteria. Further, only a subset of *X. nematophilus* and *P. luminescens* strains appear toxic to *M. sexta* (R ffrench-Constant, D Bowen, unpublished data), suggesting either that different strains lack the *tc* genes or that they carry a different *tc* gene compliment. Detailed analysis of both a strain and toxin phylogeny within, and between, these bacterial species should help clarify the likely origin of the toxin genes and how they are maintained in different bacterial populations.

Conclusions

Here we have reviewed the recent cloning of novel insecticidal toxin complex encoding genes from two different bacteria *P. luminescens* and *X. nematophilus*. These toxins appear to bear little homology to previously identified bacterial toxins and should provide useful alternatives to toxins derived from *B. thuringiensis*. Although they have similar histopathological effects on the insect midgut to other novel orally active toxins, their precise mode of action remains obscure. Future directions will include clarification of the role of extracellular proteases in toxin processing and/or activation, investigation of the export machinery used to secrete these large molecules and the relationship between the putative *Photorhabdus* and *Xenorhabdus tc* homologs.

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Patent

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